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CRISPR Phase 3 – Developing gene knockout technology in the sheep blowfly

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Executive Summary

This project has continued refining CRISPR/CAS9 technology in the sheep blowfly, building on the methods and tools developed in previous AWI funded research. We have been able to demonstrate that we can generate CRISPR mutations in short time frames, that different commercial CAS9 enzymes can be used to generate these mutations and that by producing the *white* mutant strain we will be able to build more advanced genetic tools in the blowfly. We also worked on adapting a cryonic technique for retaining and preserving important blowfly strains, such as genetically modified blowflies. Longer-term storage would provide the flexibility to pause or resume research in this area, depending on the research needs and funding capacity of AWI.

1. Introduction/Hypothesis

Clustered random interspaced short palindromic repeat (CRISPR) genome editing technology is proving to be a highly efficient and effective strategy for engineering a variety of mutations into genes in many different species. There are a variety of available CRISPR methods that allow researchers to generate many different types of mutations to study the biology of the vinegar fly (*Drosophila melanogaster*). The advances made in the sequencing of the *Lucilia cuprina* genome mean that CRISPR has the potential to be a powerful tool for the understanding of the function of genes in this pest. Establishing a routine gene editing method in *Lucilia cuprina* provides a clean and fast way to validate the potential of specific genes in the quest for the development of novel blowfly control options. This would reduce the reliance on inferences made from research conducted in other insects.

This is the third phase of a project to develop CRISPR as a tool for the manipulation of genes in *L. cuprina* (the Australian sheep blowfly), aiming to enhance the efficiency of using this technology. The initial CRISPR/CAS9 projects (ON-00315, P04500005656; ON-00516 P04500010775) successfully adapted a technology used in other species, to create gene deletions in *L. cuprina*. Flies with deletions in the *white* eye colour gene were created, leading to the establishment of a strain with an easily scored visible marker that has many downstream applications. The capacity to create gene deletions in *L. cuprina* will be of great benefit to research efforts into gaining a better understanding the biology of this ectoparasite. In this project we conducted further work on the *Orco* gene and have now generated a pure breeding fly deletion mutant, $Orco^{\Delta 88}$, that does not express the *Orco* transcript. These flies should not be able to detect odours and can therefore be used to study the role olfaction plays in the detection of suitable hosts.

2. Literature Review

There are several published genome editing techniques that employ a variety of proteins (*Cpf1* nucleases, TALENS, Zinc Finger nucleases and CAS9 nucleases), however CRISPR/CAS9 [1] stands alone for its versatility and ease of application. CRISPR/CAS9 has been utilised in a wide variety of species, and not only those that are established model organisms for study such as *D. melanogaster [2]*. CRISPR/CAS9 technology has been developed for use in pest insects such as Mediterranean fruit fly [3] and cotton bollworm [4-6] and is being used to provide detailed insights into the biology of these organisms. Another recent study has been published that used CRISPR/CAS9 in *L. cuprina* to examine a gene required for male viability [7].

One additional genetic tool that would be of value is the capacity to routinely integrate constructs into the blowfly genome at precise locations. An alternative approach to CRISPR/CAS9 to achieve this is through the use of the AttB/AttP integrase system. This first relies on an AttP landing site being integrated into the genome. To target a construct to this site, a source of integrase (RNA) and the construct carrying the AttB site are injected into flies. This has a high rate of efficiency in Drosophila (>50%) and could provide a much faster option for generating strains of blowflies to analyse particular genes of interest. By inserting different alleles of a gene into the same specific site in the genome would allow the biological impact of these alleles to be compared. Further, some of the more sophisticated tools for the manipulation of the expression of genes and proteins developed in Drosophila could be readily imported into *L. cuprina*. Thus, gene function could be tested in the blowfly itself, rather than extrapolating findings from other organisms that have significant differences in their biology, compared to the blowfly. The selection of the *white* gene as the gene targeted for the AttP insertion in this project was a strategic decision as this provides an easily identifiable phenotypic marker (flies have white instead of red eyes) that we can also use in future work, including the further development of the technology. Genetic tools utilised in *D. melanogaster* rely on this technology and have the potential to be co-opted for use in *L. cuprina* with minor modifications. This would remove the need to spend significant amounts of research time and money on developing genetic tools from scratch and allow research aimed at better blowfly control.

3. Project Objectives

- Assemble a CAS9 integration construct for *L. cuprina*
- Engineer a *L. cuprina* strain that expresses CAS9 protein
- Work on the development of a cryonic technique to preserve and store blowfly embryos.

4. Success in Achieving Objectives

Objective 1. Assemble a CAS9 integration construct for *L. cuprina*

During the project, we successfully modified constructs to include an AttB sequence that would allow precise integration of these at an AttP site in the *L. cuprina* genome. One of these constructs was also tested for integration into an AttP landing site in *D. melanogaster* and assayed for function of the codon optimised CAS9 nuclease.

Objective 2. Germline event resulting in a blowfly carrying an insertion of the AttP landing site and creation of a CAS9 expressing blowfly strain

Several different versions of the components required to generate a CRISPR/CAS9 integration event of the AttP landing site into the *white* gene were designed and the injection of combinations of these tested. This included varying the CAS9 protein sources and using two different single-stranded DNA donor templates. The desired event was detected using primers specific to the insertion event in larvae and embryos from the injections of the components, indicating the method works and that the integration of the AttP DNA does occur. Despite identifying 17 *L. cuprina* candidates that had CRISPR/CAS9 events in the *white* gene, none of these were the precise modification introducing the AttP landing site. This indicates that the frequency of the correct integration event may be low and require further rounds of injection. Only one successful integration would be required to produce a strain that could expedite all of the research that such a strain could facilitate.

Objective 3. Work on the development of a cryonic technique to preserve and store blowfly embryos

The third element of this project was to begin developing a cryopreservation technique for preserving blowfly embryos to allow for long-term storage of strategically important strains. While we have not been able to recover viable adults at this stage, we have been able to recover developing embryos through early embryonic stages, post freezing, as well as observing muscle movement in larvae pre-hatching and also on rare occasions, recovering some larvae that had hatched. These larvae died prior to progressing to the second larval instar. Different methods were tried and varied in many ways and it is encouraging that the protocol has progressed to point where we are able to observe development and occasional hatching post freezing, however further work would be required to achieve a protocol that allows the recovery of viable adults.

5. Methods & Results

Objective 1. Modifying constructs to include an AttB site.

Constructs that would express a *L. cuprina* codon optimised CAS9 protein were modified to include the 35bp AttB landing site. This additional fragment was created from annealing two oligos (Table 1). These were phosphorylated, annealed and then ligated into the constructs that had been linearised using the restriction enzyme *PstI*. The constructs were transformed into bacterial cells and these were screened for insertion of the sites using PCR.

Table 1. DNA oligos used to introduce the AttB site into CAS9 expression constructs

Oligo name	Sequence 5'-3'
PstI_AttB_35_sense	CGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCTGCA
PstI_AttB_35_anti	GGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTGCA

Positive colonies were cultured, purified and sent for sequencing. The sequence analysis indicated that AttB sites had been integrated successfully into the constructs (Figure 1).

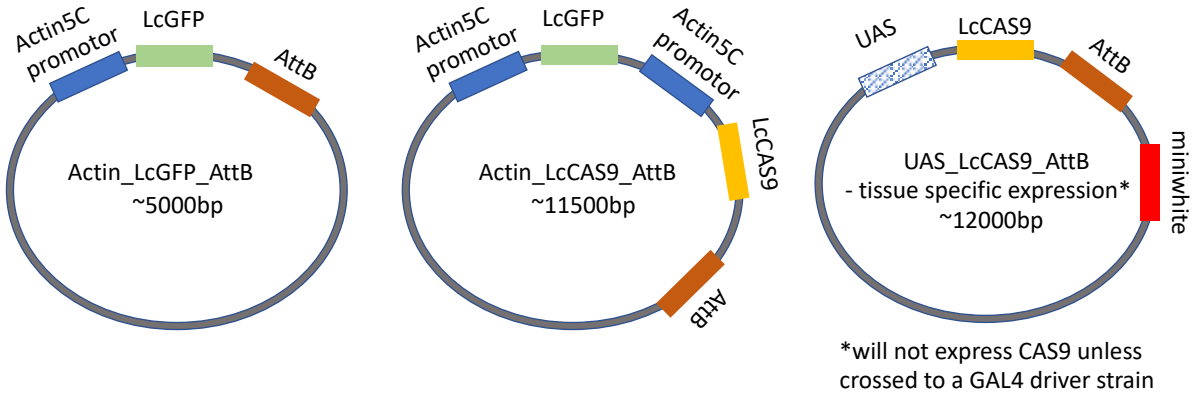

Figure 1. Constructs created that would allow their specific integration at an AttP landing site. Actin_LcGFP_AttB is a reporter construct to test the AttB landing site and to mark regions CAS9 protein will be expressed. Actin_LcCAS9_AttB is designed to express CAS9 throughout the fly and also carries the GFP marker.

The UAS_LcCAS9_AttB landing construct was also generated using *L. cuprina* codon optimised CAS9 nuclease (LcCAS9). This construct was injected into *D. melanogaster* to create transgenic flies we used to confirm that the construct would insert. This was shown to be the case, with transgenic flies recovered from the injections. We also crossed the flies to test that the nuclease was functional. We observed cutting events indicating that the construct produces LcCAS9 and this protein can be targeted to cut specific DNA sites in the presence of guide RNAs (Figure 2.). Therefore, the codon optimized CAS9 is likely to function when we are able to successfully transform the construct into the blowfly.

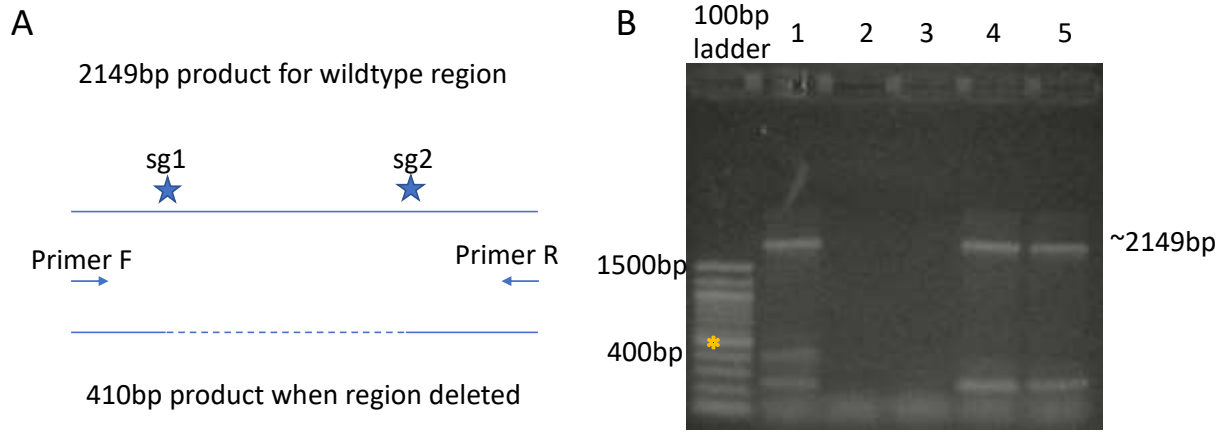

Figure 2. Testing confirms that the *L. cuprina* version of a codon optimised CAS9 protein is functional in *D. melanogaster*. Panel A. Amplification using Primer F and Primer R should produce a 410bp band when correct CRISPR/CAS9 cutting occurs, as opposed to a 2149bp band when there is no cutting. Panel B. Agarose gel visualising PCR products from larvae. We see both these bands in the experimental (lane 1) as expected as these larvae have both the LcCAS9 expressed in neuronal cells and sgRNAs that will guide the protein to the correct DNA sites. The deletion band is marked with an *. Lanes 2 and 3 are negative no template controls, lanes 4 and 5 are the two parental lines that carry either the sgRNAs or the LcCAS9 and do not have cutting events.

Objective 2. Integration of the AttP landing site at the *white* locus of *L. cuprina* and creation of a CAS9 expressing blowfly strain.

Injections were carried out as previously reported using a CAS9 protein that was preloaded with sgRNA that would target cutting to the *white* gene at the sg3A site that had previously been used to generate the *white* deletion strain. We designed a screen for the CRISPR/CAS9 mediated insertion of the AttP landing site that would lead to a disruption of the *white* gene (Figure 3). Cages were set up of surviving injected flies that were crossed to white-eyed flies of the opposite sex.

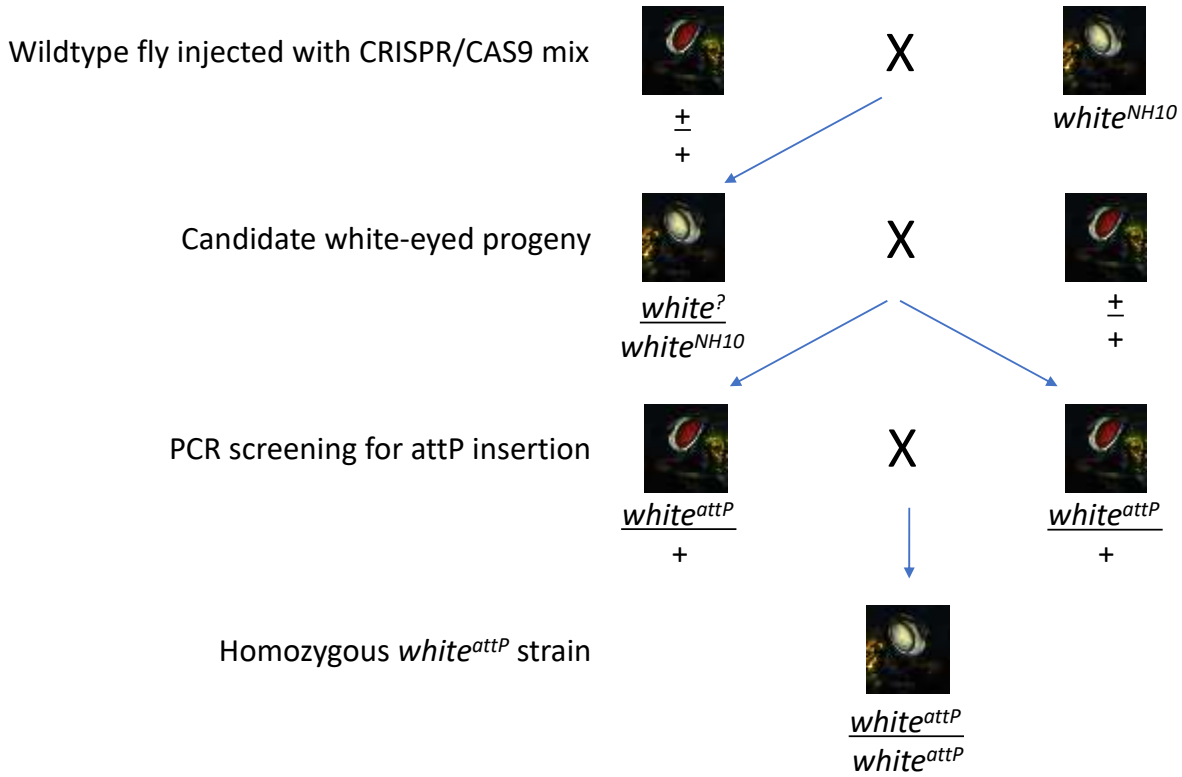

Figure 3. **Crossing scheme to isolate flies carrying an AttP insertion in the *white* gene.** Injected flies were crossed to the strain *white*^{NH10}, that carried a large deletion at the *white* genomic locus. This meant that any inherited CRISPR events (denoted by *white*) would be visible in the next generation, both mutation and specific insertion events, eliminating the requirement to screen every one of the progeny for potential events. As any disruption of the *white* gene leads to the white-eye phenotype, a diagnostic PCR was prepared that would allow the detection of the desired AttP landing site insertion.

We performed five rounds of injections, attempting to insert the AttP landing site that had surviving adults and the results from this work are summarised in **Table 2**. Variations were made to the oligo template sequence and also the CAS9 protein source in an attempt to optimise the protocol for an insertional event. The use of the *white* background for detection of events was successful and we had three out of five rounds of CRISPR/CAS9 injections that generated inherited events, recovering a total of 17 individual flies. The rate of inherited CRISPR mutations from these was between 0.28-0.95%.

Table 2. CRISPR/CAS9 injection results for AttP sequence insertion.

Round	Protein source	sgRNA:oligo combination	# adult survivors	# progeny scored	# CRISPR events	% events
1	AltR_spCAS9 IDT	sg3A/attP	7	200	0	-
2	AltR_spCAS9 IDT	sg3A/attP	80	840	8	0.95
3	AltR_spCAS9 IDT	sg3A/attP_2019	30	760	0	-
4	AltR_spCAS9 IDT	sg3A/attP_2019	35	1070	3	0.28
5	Spy_Cas9 NEB	sg3A/attP_2019	40	1350	6	0.44

Rounds 2, 4 and 5 had a total of 17 white-eyed survivors that were analysed for an insertion of the AttP landing site. Of these, no flies were detected that had an amplified DNA fragment of the correct size. This indicated that the flies isolated from this were CRISPR mutation events, rather than specific insertion events. To determine if the components are functional and that the insertion can occur, we sacrificed some of the injected larvae and were able to detect the presence of the correctly sized DNA fragment, predicted to arise from insertion at the correct locus (Figure 4). These proved to be insertions in somatic cells and were, thus, not inherited.

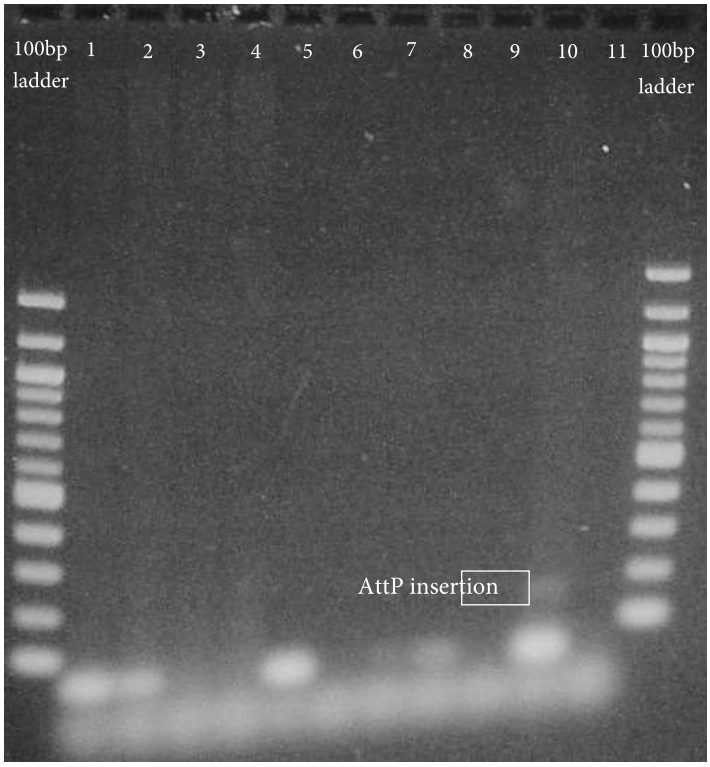

Figure 4. Diagnostic PCR visualised on agarose gel confirming AttP integration occurs in injected larvae. DNA from candidate flies (lanes 1-9) from the injected larvae sample (lane 10) and wildtype control (lane 11) was tested. The feint band in the white box indicates AttP integration events. While we could detect this in the injected flies, there were no germline integration events recovered in any of the white-eyed adult CRISPR/CAS9 mutants we isolated.

Objective 3. Work on the development of a cryonic technique to preserve and store blowfly embryos

Testing was conducted using a large range of conditions in an attempt to develop a technique for cryonic storage of embryos (Table 3). Conditions were adapted from a number of reported methods [8-10]. We have been able to observe development, and in some cases hatching, of larvae using the conditions listed below from embryos that were frozen in liquid nitrogen. More testing with variations to the method will be required for this to become a practical solution for long term storage and recovery of *L. cuprina* strains.

Table 3. Summary of cryonic preservation conditions tested

Egg Collection	Plate Incubation	1.8M EG incubation	Vitrification concentration	Vitrification timing	Mouth hook %
30	6h20min	20	34%	6	0
30	6h	20	37%	12	0
30	6h	20	37%	6	0
30	6h	20	37%	12	0
30	6h	20	37%	12	0
30	6h	20	37%	12	0
30	6h	20	37%	6	0
30	6h	20	37%	12	0
30	6h20min	20	37%	8	0
30	6h20min	30	37%	15	0
30	6h20min	30	37%	20	0
30	6h20min	40	37%	10	1
30	6h	20	37%	6	3
30	6h20min	40	37%	6	4
30	6h20min	30	37%	15	5
30	6h20min	40	37%	10	4
20	6h40min	20	37%	6	10
30	6h20min	20	37%	6	24
❖ 30	**❖ 6h20min**	**❖ 30**	**❖ 37%**	**❖ 12**	**❖ 33**

Final conditions for the protocol that led to the highest level of development after recovering embryos after freezing are in the last row of Table 3. We also observed muscle movement and some larvae that had hatched from the embryos, indicating that the protocol is getting closer to the goal of recovering viable flies after freezing. Based on our testing results, the current state of a protocol for preserving embryos is described below.

<u>Method under development for cryonic storage of *Lucilia cuprina* embryos</u>

Egg collection and Incubation time

1. Adult flies were permitted to lay eggs on a piece of beef for 30 min.
2. The eggs were incubated at 37°C for 6h20min on grape juice plates.

Embryo Permeabilization

3. Break up egg masses by placing in 1.0% NaOH for 2 min and agitating.
4. Rinse eggs with running tap water for 1 min.
5. Embryos were dechorionated for 90s in a 6% solution of sodium hypochlorite (bleach).
6. Rinse eggs with running tap water for 3 min.
7. Agitate in 2_propanol for 10s.
8. Air dry for 3 min or until is completely dried.
9. Agitate in hexane for 30s to remove the lipid layer associated with the membrane of embryos.
10. Air dry for 1 min or until is completely dried.
11. Agitate with a Plastic Pasteur pipette to break up any clumps in Drosophila Ringer's solution for 2 min.
12. Under a Stereo Microscope check the current developmental stage of embryos that were dechorionated and permeabilized.

Loading Embryos with Ethylene Glycol (EG)

13. Place embryos in 1.8M Ethylene Glycol in Schneider's insect cell culture medium for 30 min at room temperature.

Dehydration in Vitrification Solution

14. Place embryos in 37% wt EG + 5.4 wt % PEG + 15.5 wt % trehalose + 45.1 wt % SS for 12 min on ice. They should look similar to those depicted in Figure 5.

Figure 5. Dehydrated *Lucilia cuprina* embryos in vitrification solution

Vitrification and Storage in Liquid Nitrogen

15. Transfer the embryos to a 25mm Whatman nuclepore track-etch polycarbonate membrane by touching the eggs floating on the solution's surface with the membrane.
16. Blot the reverse side of membrane with adhering embryos to remove the excess vitrification solution.
17. Hold the membrane above liquid nitrogen vapor for 1 min.
18. Quickly plunge membrane with embryos into liquid nitrogen. When vitrification occurs, the embryos become glassy, light yellow in colour. Embryos turning snow white indicates that an insufficient amount of cryoprotectant has been loaded into the embryos.
19. Transfer membrane with vitrified embryos to suitable container for liquid nitrogen storage.

Recovery from Liquid Nitrogen

20. Remove membrane with adhering embryos from liquid nitrogen storage.
21. Hold membrane above liquid nitrogen in vapor for 1 min.
22. Quickly plunge membrane into solution containing 0.5M trehalose and 10% FBS (fetal bovine serum) in SS (Schneider's cell culture medium) for 2 min at room temperature.
23. Wash membrane and embryos at least three times with 10% FBS in SS at intervals of 10 min and remove the membrane.
24. Leave embryos in a petri dish with 10% FBS in SS overnight for hatching and then place larvae on larval diet.

Confirmation the *Orco* gene is not expressed in a CRISPR/CAS9 engineered deletion strain, *Orco$^{\Delta 88}$*.

We also continued to isolate a strain carrying a deletion of the *Orco* gene that should be incapable of detecting odours. After confirming the flies were homozygous for the deletion, we tested for expression of *Orco* at the RNA level. RNA was extracted from the olfactory organs (antennae and maxillary palps) of the *PBPop* wildtype strain and the *Orco$^{\Delta 88}$* strain. Using both oligo$_{dT}$ and Random hexamer primers to generate cDNA, we were only able to detect the presence of *Orco* expression in the *PBPop* strain and confirmed that there is no expression of *Orco* in our deletion strain (Figure 6.). This strain can now be tested for a lack of response to odours and once confirmed, it can be used to ascertain the contribution of odour relative to other senses such as sight and taste for the level of attraction to sheep.

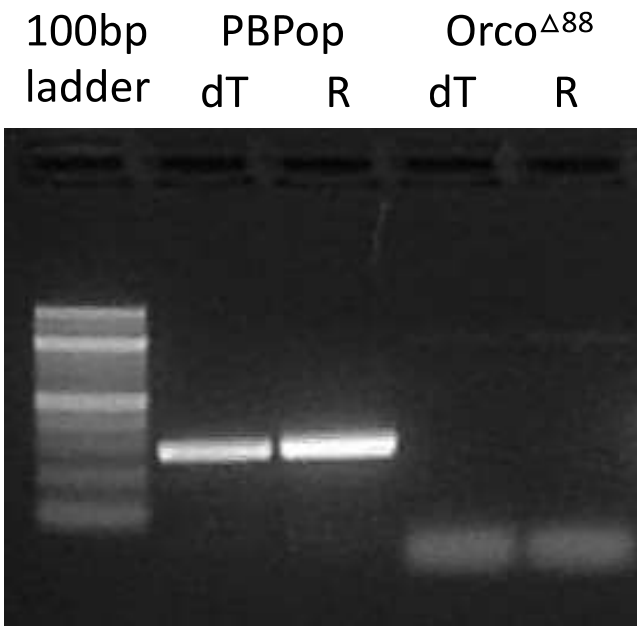

Figure 6. Confirming the *Orco*$^{\Delta 88}$ strain does not express mRNA from the *Orco* gene. Primers were used to detect the *Orco* mRNA and would produce a 280bp fragment. The presence of this was clearly observed for the wildtype *PBPop* strain but this was absent in the *Orco*$^{\Delta 88}$ strain indicating the deletion has removed expression of the gene.

6. Discussion

The CRISPR/CAS9 method has been established in the blowfly and we are now able to demonstrate the capacity to regularly isolate gene editing events. While a CAS9 expressing strain has not been isolated, we were able to produce a codon optimised construct and demonstrate it functions to mediate specific gene editing under the control of guide RNAs in *D. melanogaster*. In future, we plan to establish a CAS9 strain for use in blowfly research, however maintaining it has associated costs that will require improvements to the cryonic preservation method. We also managed to produce a homozygous *Orco* deletion strain that will be of value to future studies examining the role of odour in *L. cuprina* in detecting and parasitising sheep.

The cryonic preservation protocol will be an economical option for preserving and storing embryos of blowfly strains, and there are a number of circumstances where this would have scientific value, in addition to averting high ongoing maintenance costs of strains. This would include the preservation of CRISPR mutants and archiving blowfly samples from the field, allowing evolutionary changes in blowfly populations (e.g. insecticide resistance) to be identified. We believe that the protocol is close to succeeding and that slight modifications to additional variables such as time of development and temperatures used for this incubation period might help to enhance the survival to the point that it will be possible to recover strains stored in liquid nitrogen using this method in the future.

7. Impact on Wool Industry – Now & in 5 years' time

The use of the CRISPR/CAS9 technique in *L. cuprina* has been established and enables scientists to conduct targeted genetic manipulation in their research into blowfly biology. This project has helped us to improve the method further, as demonstrated by the now regular recovery of CRISPR/CAS9 events, albeit at a frequency that can still be improved. Our research will continue to develop and use this method, and it will be particularly valuable in helping us pursue research into novel blowfly specific genes and their roles in blowfly parasitism of sheep. Without this capacity we would not be able to conduct this work. Another benefit arising from routine use of CRISPR is that it enhances the attractiveness of blowfly research to the next generation of researchers. We are now able to offer student projects in this space and this has led to the recruitment of new students working on blowfly biology. These students may otherwise have been lost to research on other organisms. This technology will be of great value to many other labs and a diverse range of research projects working on both fundamental biological questions and the development of blowfly control strategies including sterile insect technique and gene drive. There are likely to be many applications of the technology that will be of interest to the Wool Industry and it is a space that needs to be regularly monitored given how quickly the technology and the regulatory landscapes are shifting.

8. Conclusions and Recommendations

The project has developed the CRISPR/CAS9 technology to the point that it can now be used routinely in the blowfly. Despite not being able to recover a strain with a successful integration of the AttP site, we were able to detect predicted integration events were occurring and were able to optimise the detection of these using resources generated in prior projects. The lack of a correct event indicates that the frequency of specific integration is low and that greater numbers of flies will need to be examined to identify events in the future. The results of this and prior studies will be presented at the 2019 Australian Society for Parasitology meeting, and methods will be published later this year. CRISPR/CAS9 is now at a point where it is being developed further by us and other research groups. Another research group (led by Max Scott, North Carolina State University) has published their use of CRISPR to examine a specific gene required for male viability in *L. cuprina* [7] and more groups are likely to follow given that this technology will be a very effective way to conduct the research in the blowfly. With the continuing debate around classification of GMOs and uncertainty about their use, regularly reviewing the technological and regulatory aspects of gene editing, both domestically and internationally, to remain up-to-date is likely to be of value. This will allow future projects in this area to be effectively evaluated, with consideration not only given to cost-benefits, but also to the likelihood that end products would be able to satisfy and pass regulatory hurdles for their use.

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10. List of Abbreviations/Glossary

CRISPR (Clustered random interspaced short palindromic repeat)

Orco (Odorant co-receptor)

PCR – polymerase chain reaction